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(57) Abstract

Replication-defective viruses and means for intracellular replication thereof are described which are useful for gene therapy. Human cells can be changed into recombinant replication-defective virus particle-producing cells by the simultaneous delivery to those cells of two different nucleic acids: the first being a replication-defective viral genome, the second being a nucleic acid that complements the viral sequences deleted from the first nucleic acid so as to result in the production of new infective virus. The first nucleic acid can be delivered by the replication-defective virus itself or, as a nucleic acid that is not part of the virus. In a preferred embodiment, the replication-defective virus includes elements to maintain the two nucleic acids in combination during transduction. Examples of preferred viral sources are adenoviruses, herpesvirus, retroviruses, and adeno-associated viruses. Nucleic acids useful for gene therapy include those that code for proteins used to identify cells infected with the recombinant virus, those that encode for proteins that function to kill cells containing the viral genome, or that encode for therapeutic proteins that will serve to treat a pathophysiologic condition within the body.

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COMPOSITION AND METHODS FOR CREATING SYNGENEIC RECOMBINANT VIRUS-PRODUCING CELLS

Background of the Invention

The present invention is in the general field of gene therapy, and is in particular in the area of engineering viral vectors for transduction of normal cells.

"Gene therapy" refers to the treatment of pathologic conditions by the addition of exogenous nucleic acids to appropriate cells within the organism. Nucleic acids must be added to the cell, or transduced, such that they remain functional within the cell. For most gene therapy strategies, the new nucleic acids are designed to function as new genes, i.e., code for new messenger RNA that in turn codes for new protein. As originally conceived, gene therapy was directed towards monogenetic disorders like adenosine deaminase deficiency and cystic fibrosis. It has become abundantly clear that gene therapy might also be helpful in polygenetic somatic disorders like cancer.

The rapid implementation of gene therapy in human trials has been made possible by the development of relatively efficient means of adding new nucleic acids to cells, a process generally referred to as "gene transduction". The clinically applicable gene transduction methods fall into one of three categories: a) cationic lipids (b) molecular conjugates (c) recombinant viruses. These different means of accomplishing gene transduction have been recently reviewed (Morgan 1993 Ann. Rev. Biochem. 62:191.; Mulligan 1993 Science 260:926; Tolstoshev 1993 Ann. Rev. Pharm. Toxicol. 32:573

Although the three major groups of gene transduction methodology are relatively efficient,

the percentage of target cells that can be transduced *in vivo* remains relatively low. For the treatment of conditions requiring a higher percentage of gene transduction, new technologies for increasing the percentage of transduced cells would be a very useful advance.

One pathophysiologic condition where transduction efficiency has been shown to be a limiting factor is cancer. Some strategies for cancer gene therapy entail the addition of toxin genes, or other genes deleterious to cancer growth, to the tumor mass. These approaches have been facilitated by the use of toxin genes that kill not only the transduced cells, but also adjacent cells by a "bystander effect". The herpes simplex virus thymidine kinase + ganciclovir system exemplifies the desired bystander effect (Freeman 1992 Cancer Research **53**:5274). In this case, the viral thymidine kinase gene converts the prodrug, ganciclovir, into a phosphorylated nucleoside analog that blocks DNA replication and thereby further growth (Paul 1992 Amer. J. Med. Sci. **304**:272). The phosphorylated ganciclovir bystander effect has recently been shown to be the consequence of gap junction - mediated transfer of the toxin to adjacent cells (Stambrook 1993 Cancer Gene Therapy **1(suppl)**:1).

However, the benefit of the bystander effect in treating animal models of solid tumors has required the administration of viral producing cell lines to yield adequate transduction with the consequence of measurable reductions in tumor mass. This strategy was first reported by Culver et al for the treatment of brain tumors engrafted in nonhuman primates (Culver 1992 Science **256**:1550). In this report, a murine cell line that produced a recombinant retrovirus containing a viral thymidine

kinase gene was administered to the engrafted tumor mass, the concept being that the tumor cells would be continually exposed to new virus over an extended period of time. The animals were subsequently treated with ganciclovir and demonstrated dramatic reductions in tumor mass. This concept has now been extended to liver tumors and subcutaneously engrafted tumors (Caruso 1993 Proc. Natl. Acad. Sci. USA **90**:7024; Freeman 1993 Cancer Res. **53**:5274).

The producer cell line approach does have important limitations. First, the producer cells are xenografts of immortalized cells that may be quickly eliminated by host immune mechanisms. Second, this method relies on the existence of stable producer cell lines that are available only for recombinant retroviruses, but not for other recombinant viruses. The recombinant adenoviruses, adeno-associated viruses, and herpes viruses are all produced by lytic infections of their corresponding "packaging" cell lines. Thus, these viruses cannot be utilized for this type of cancer therapy. Third, it has become apparent in the first human trial of recombinant retroviral producer cell treatment of brain cancers that multiple injections were required to produce partial responses in 5 of 8 patients treated (Ram 1993 Cancer Gene Therapy **1(suppl)**:1). This has prompted these investigators to pursue a strategy that utilizes multiple injections of the tumor mass that is of unproven utility that will not be applicable to other sites of tumor mass where the tumor location cannot be as precisely fixed as is possible within the rigid confines of the human

The clinical applications of gene therapy would be greatly advanced by the development of new

approaches to achieving high levels of gene transduction *in vivo*. A particularly valuable approach would be one that provides for the continuous production of new recombinant vector that is applicable to multiple vector systems and does not require the administration of xenogeneic cells.

It is therefore an object of the present invention to provide vectors for gene therapy and methods for use thereof not requiring xenogeneic cells.

It is a further object of the present invention to provide improved defective viral vectors that can be co-transduced with elements for intracellular reproduction of the defective viral vectors, which are capable of infecting other cells.

Summary of the Invention

Replication-defective viruses and means for intracellular replication thereof are described which are useful for gene therapy. Human cells can be changed into recombinant virus-producing cells by the simultaneous delivery to those cells of two different nucleic acids: the first being a replication-defective viral genome, the second being a nucleic acid that complements the viral sequences deleted from the first nucleic acid so as to result in the production of new infective virus. The first nucleic acid can be delivered by the replication-defective virus itself, or as a nucleic acid that is not part of the virus. In a preferred embodiment, the replication-defective virus includes elements to maintain the two nucleic acids in combination during transduction.

Examples of preferred viral sources are adenoviruses, herpesvirus, retroviruses, and adeno-

associated viruses. Nucleic acids useful for gene therapy include those that code for proteins used to identify cells infected with the recombinant virus, those that encode for proteins that function to kill cells containing the viral genome, or that encode for therapeutic proteins that will serve to treat a pathophysiologic condition within the body. Commonly, the second nucleic acid sequence provides sequences that in a trans configuration enable the first nucleic acid to replicate and be packaged into new, replication-defective viral particles that can also contain other nucleic acids that are useful for gene therapy. A preferred example of a linking means for the two nucleic acids is to conjugate on the surface of the defective virus containing the first nucleic acid highly charged polyamino acids, such as polylysine, which binds ionically to the second nucleic acid.

Brief Description of the Drawings

Figures 1A and 1B are schematics of the infection and replication processes of replication-competent virus (Figure 1A) and replication-incompetent, or defective, virus (Figure 1B).

Figures 2A-2D are maps of the complementary adenoviral nucleic acid sequences.

Figure 2A is a map of the AdCMVlacZ viral genome. Hatched bars represent the conserved human type 5 adenovirus sequences with map units indicated below. CMV promoter sequences ("CMV"), bacterial lac Z sequences ("lac Z"), and SV40 polyadenylation signal ("pA") were inserted as a cassette in place of the deleted E1A and E1B sequences.

Figure 2B is a map of pE1[illegible]. Adenovirus [illegible] type [illegible] nucleic acids are shown. Ad [illegible] with nucleotide numbers above and map units below the

indicated fragment. The Ad 5 nucleic acids were ligated into the multiple cloning site of pUC 13 represented by hatched bars at the indicated restriction endonuclease sites. "E" = *Eco* RI, "B" = *Bst* 1107 I, "H" = *Hinc* II.

Figure 2C is a map of the AdCMVluc viral genome. Black bar represents the deleted E1 region sequences into which was inserted the luciferase expression cassette consisting of the CMV promoter ("CMV"), firefly luciferase coding sequence ("luc"), and an SV40 polyadenylation signal ("pA").

Figure 2D is a map of the adenoviral E1 region to indicate the viral sequences inserted into the replication-enabling plasmids pE1A and pE2206.

Figure 3 is a graphic representation of the observed cytopathic effects produced by lysates or supernatants from cells cotransduced with AdCMVlacZ and plasmid DNA. Ordinate = qualitative amount of cytopathic effect 48 hrs after addition of the supernatants or lysates, abscissa = times of lysate or supernatants after cotransduction. Key indicates the plasmid cotransduced with the adenovirus. The pUC 13 groups were observed up to 96 hrs after supernatant or lysate addition without any cytopathic effect.

Figure 4 shows the luciferase transfer capacity in the supernatants of HeLa cells cotransduced with DOTAP/DNA complexes containing AdCMVluc DNA and pE1A plasmid DNA. Shown are the results 6 days after exposure to the DOTAP/DNA.

Figure 5 shows the luciferase activity present in engrafted PC-3 tumors 10 days after they were injected with either AdCMVluc and pE1A, or for [illegible] AdCMVluc and pUC 13.

Figures 6A and 6B are maps of the retroviral nucleic acids.

Figure 6A is a map of pBAG. "LTR" = long terminal repeat, "β-galactosidase" = bacterial lac Z gene, "neo" = neomycin resistance gene, "pbr origin" = plasmid origin of replication. Not to scale.

Figure 6B is a schematic of retroviral sequences within the pPAM3 plasmid. Open blocks at left indicate the 5' LTR sequences, "SD" = splice donor site, "SA" = splice acceptor site, "gag, pol, env" = retroviral coding sequences, hatched bar = SV40 polyadenylation signal. (Reproduced from (Miller 1986 Molecular and Cellular Biology **6**(8):2895.).

Figure 7 is a graphic representation of the titers of retrovirus in supernatants of cells cotransduced with pBAG and pPAM3. Supernatants were collected 72 hrs post-transduction with the complementary retroviral vectors. Ordinate = titer as number of viral particles per ml using A549 as indicator cells, abscissa = amounts of the 2 plasmids used to make the molecular conjugates. The cell type cotransduced with the two plasmids were A549 (circles) and PC-3 (triangles).

Detailed Description of the Invention

As used herein, "replication" is the process of producing new viral particles that are capable of infecting other cells. "Infection" is the process of a viral particle binding to a specific cell surface receptor resulting in the release of the viral genome into the nucleus where the viral genome directs the production of new proteins.

As shown by Figure 1A, a replication-competent virus infects a cell when the viral particle binds to a receptor on the surface of the cell and is transported into the cytoplasm and then

into the nucleus. Once in the nucleus, the complete viral genome is transcribed, new viral proteins are synthesized, and new viral particles are made and released.

As shown by Figure 1B, in the case of a replication incompetent virus, the viral particle containing an incomplete genome also binds to a cell surface receptor and is transported through the cytoplasm into the nucleus. However, since the viral genome is incapable of either being transcribed, or directing synthesis of all viral proteins required for packaging, infection with a replication-defective virus does not lead to new viral particle production.

The production of syngeneic, replication-defective, recombinant virus-producing cells requires the following steps. The fundamental starting point is the requirement for a replication-defective virus that can produce new replication-defective virus in the presence of additional genes provided in a trans configuration. Although the specific designs of these different viruses vary, as a general rule the recombinant viral genome has some of the native viral genes required for replication deleted, which may be replaced by new genes of interest. Typically, the replication-defective virus includes the 5′ and 3′ LTRs and packaging signal sequences. This replication-defective virus is generally referred to herein as the "first nucleic acid sequence".

The second step is the design of complementing genomes that will be co-delivered to the target cells in order to produce new recombinant virus. These complementing genomes must be present in the cells in which new recombinant virus is to be produced but should preferably not recombine with the defective virus

to produce replicating virus. The complementing genome is generally referred to herein as the "second nucleic acid sequence".

As part of either the first and/or second steps, the biologically active genes of interest which are to be delivered and expressed in the target cells are incorporated into either, or both, the first and second nucleic acid sequences, most preferably the first nucleic acid sequence.

An optional third step is to provide means for co-delivering the first and second nucleic acid sequences.

The final step is to co-deliver the first and second nucleic acid sequences. This can be accomplished by multiple, widely available gene transduction methods. One then must establish that co-delivery of the first and second nucleic acids results in the production of new recombinant virus which is infectious and capable of expressing the incorporated genes of interest in the target cells. The presence of new recombinant virus can be assayed in the supernatant of the cells transduced several days later, or within lysates of the transduced cells. The identity of the new virus can be established by the presence of marker genes, or marker gene products, within the recombinant virus genome.

Replication-Defective Virus ("RDV")

Many replication-defective viruses, particularly derived from retroviruses, adenoviruses, adeno-associated viruses, and herpes viruses, have been described in the literature and are available from sources such as the American Type Culture Collection, Rockville, Maryland. These viruses are characterized as missing all or a portion of one or more genes essential for replication of the virus. In an appropriate cell

line, however, they are capable of forming infectious virus particles. The viruses useful in the methods described herein are those capable of infecting and replicating in mammalian cells, although they may infect certain cell types preferentially.

Retroviruses are generally defined as a family of eukaryotic viruses that replicate through a DNA intermediate, as described by Panganiban 1985 Cell 42:5.

Herpes virus are classified based on a characteristic virion architecture that includes an icosadeltahedron forming the capsid with two-fold symmetry surrounding the tegument that surrounds the DNA genome, as described by Roizman in Human Herpesviruses 1993 (Raven Press) pp. 1-9.

Adenovirus are nonenveloped viruses containing a double stranded DNA genome with characteristic antigenic properties and DNA homology, as described by Wadell 1984 Current Topics in Microbiology **110**:191.

Parvoviridae are a family of DNA, non-enveloped animal viruses containing a single stranded DNA genome encapsulated within an icosahedral protein coat composed of three proteins with overlapping amino acid sequences. The family includes three genera that includes (i) parvoviruses (ii) adeno-associated viruses (AAV) that usually require coinfection with adenovirus (iii) densoviruses which multiply in insects, as described by Berns 1990 Microbiological Reviews **54**:316.

Recombinant retroviruses are generally made by manipulating the proviral form of the virus, i.e., the double stranded DNA copy of the viral RNA genome. The starting virus is commonly the Maloney murine leukemia virus that is available

from ATCC, Rockville, MD. Examples include VR-861, VR-860, VR-590, and VR-589. The proviral DNA is placed within a plasmid to permit amplification of the DNA in bacteria. Once this has been accomplished, the gag, pol and env genes are excised by appropriate restriction endonucleases to leave the 5′ and 3′ long terminal repeats (LTRs), and the packaging signal sequences that are immediately 3′ to the 5′ LTR. Multiple, detailed descriptions of these constructions, as well as permutations of this general scheme, are clearly described in the scientific literature, for example, by Cepko 1984 Cell **37**:1053; Hwang 1984 J. Virol. **50**:417; Yu 1986 Proc. Natl. Acad. Sci. USA **83**:3194; Armentano 1987 J. Virol. **61**:1647; Yee 1987 Proc. Natl. Acad. Sci. USA **84**:5197; Hawley 1989 Nuc. Acids Res. **17**:4001; Miller 1989 BioTechniques **7**:980.

Recombinant adenoviruses are generally made by manipulating the adenoviral DNA within plasmids to permit amplification of the DNA in bacteria. To date, the human serotypes 2 and 5 have been used for gene therapy purposes, largely because their biology and genomes have been most extensively characterized. The wild type adenovirus type 2 and 5 are readily available from the ATCC, Rockville, MD: Type 2, VR-846 and VR-1079; Type 5, VR-5 and VR-1082. Most commonly, the E1A region is deleted using convenient restriction endonuclease sites within the E1A region. Often, a portion of E3 is also deleted by restriction endonuclease addition so as to permit the insertion of a larger piece of foreign DNA while still satisfying the size constraints required for packaging into new viral particles. The details of adenoviral vector constructions are widely described in the literature, for example, by

Berkner 1984 Nuc. Acids Res. **11**:6003; Ghosh-Choudhury 1987 Biochem. Biophys. Res. Commun. **147**:964; Gilardi 1990 FEBS **267**:60; Mittal 1993 Virus Res. **28**:67; Yang 1993 Proc. Natl. Acad. Sci. USA **90**:4601.

Traditionally, these replication-defective viruses have been replicated through the use of packaging cell lines. These cell lines contain, at a minimum, the nucleic acid sequence obtained from replicating virus that will complement in trans the replication-defective viral genome, resulting in new replication-defective virus. A packaging cell line is made by stably introducing the missing viral genes that are required for replication. Recombinant virus is made by introducing the recombinant viral genome into the packaging cell line, the viral genome (minus the genes present in trans that complement) is replicated and packaged into viral particles that can infect any cell type with the required viral receptor. However, since the virus is still replication-defective, the viral particles are incapable of directing the production of new virus.

It has not previously been established whether other elements in the specific packaging cell lines might contribute to the production of virus in unpredicted or unexpected ways. For example, it is helpful to briefly consider the 293 cell line used for recombinant adenovirus production. These cells were made by the introduction of randomly sheared adenoviral DNA that has been only partially characterized (Graham 1977 Journal of General Virology **36**:59; Aiello 1979 Virology **94**:460). Although it has been shown that these cells contain E1A genes, and are capable of packaging E1A deleted recombinant adenoviruses, it was never established whether other sequences

also present played a role, whether the copy number of the sheared viral nucleic acid sequences present in each cell was critical to the utility of this specific cell line, or whether the specific parent cell type contributed to the virus production. Similarly, although the retroviral nucleic acids used for establishing the PA317 packaging cell line were precisely identified (Miller 1986), it was unclear whether these sequences had to be stably integrated within a cell line before the addition of the recombinant viral genome, i.e., it was never established that co-delivery of the two complementing genomes would result in the production of recombinant viral particles.

Some recombinant viral vectors are used to produce recombinant virus particles by coinfection with a helper virus, but the specific helper virus genes that are required to replication-enable the viral vector have not been defined. Specific examples of such vectors include the adeno-associated virus vectors described by Nahreini 1993 Gene **124**:257; and Samulski 1989 J. Virol. **63**:3822, and herpes vectors, described by Geller 1988 Science **241**:1667; and Breakfield 1991 New Biol. **3**:203.

A replication-defective adenoviral genome was delivered by viable virus in the specific examples described below. The same nucleic acids alternatively could be delivered in a plasmid form, since the methods for propagation of part (Berkner 1983 Nucleic Acids Research **11**:6003), or all of the adenoviral genome (Graham 1984 EMBO Journal **3**:2917) within plasmid DNA have been previously described.

Genes required for replication of replication-defective virus

In general, it is a straightforward matter to supply the genes missing in the replication-defective virus (RDV) in order to provide a second nucleic acid sequence that operates in trans with the RDV to replicate the RDV. Although the second nucleic acid sequence can include nucleic acid sequence also present in the RDV, it is preferred that there not be overlapping sequence since this can cause an undetermined amount of recombination, leading to wild type virus capable of replicating. This is not in itself necessarily a problem, however. As referred to herein, the second nucleic acid sequence includes at a minimum the genes required for replication of the replication-defective virus and means for amplification thereof. It may optionally include genes of interest, such as marker genes, suicide genes, and therapeutic genes, as described in more detail below. Although referred to as a "sequence", the parts of the second nucleic acid sequence can be present in one or more molecules, usually plasmids, preferably a single plasmid.

In general, the genes required for replication ("GRR") can be obtained by amplifying some or all of the viral nucleic acids present in a packaging cell line useful for replicating the RDV. The GRR can also be obtained by excision from virus that is capable of replication. For example, in the case of recombinant adenovirus, the viral nucleic acids that contain the E1A region are excised, subcloned and amplified by routine techniques. These nucleic acids complement the deleted E1A sequences in common recombinant [illegible] cell lines, such as the [illegible] cell line, for the

purpose of replicating the viruses. In the case of recombinant retrovirus, the nucleic acids that are used to make the packaging cell line are obtained in plasmid form and amplified with the intent of co-delivery with the recombinant retroviral vector plasmid.

The second nucleic acid sequence must be provided in a form that can be amplified, although it is preferably not in a form that infects cells other than the targeted cells which are transduced with the first nucleic acid sequences. By using a second nucleic acid sequence which is limited to defined cells, replication and infection of cells can be controlled. This is particularly important in the case where the genes of interest incorporated with the first and/or second nucleic acid sequences result in the death of the host cells. The most preferred form therefore is a plasmid. However, the second nucleic acid could also be provided within a recombinant virus, preferably one utilizing different receptors than a virus containing the first nucleic acid. Alternatively, the replication-enabling plasmid could also be provided in the form of ribonucleic acid. In all cases, the second nucleic acid must include genes with the necessary transcription activating and terminating elements necessary to transcribe mRNA that can be translated into proteins capable of enabling replication.

In the preferred embodiment where the means for amplification of the GRR is a plasmid, the plasmid promoter should allow replication in the cells to be transduced with the second nucleic acid sequence. The promoter may be cell type or tissue type specific as another means for controlling which cells are targeted

The E1A genes required for replication of adenovirus are provided in the pE1A plasmid as described in the following examples. The construction of this plasmid is described in detail in the example using pEco RIA plasmid described by Berkner 1984 Nuc. Acids Res. However, the E1A fragment or other complementing fragments that might be needed for replication-enabling other adenoviral vectors, could easily be derived from adenoviral DNA that is harvested from adenovirus-infected 293 cells, or other adenoviral-permissive host cells.

The genes required for replication of retrovirus are provided in the pPAM3 plasmid described in the following examples. The construction of this latter plasmid is described in detail by Miller 1986 Mol. Cell. Biol. **6**:2895. This description could be applied by one of ordinary skill in the art to construct an equivalent plasmid from the murine leukemia virus proviral DNA.

Genes to be incorporated into First or Second Nucleic Acids

Nucleic acids useful for gene therapy include those that code for proteins used to identify cells infected with the recombinant virus, those that encode for proteins that function to kill cells containing the viral genome, or that encode for therapeutic proteins that will serve to treat a pathophysiologic condition within the body.

The sequences that encode for many of these proteins are known and published in the literature. Representative marker genes include those described in detail in the following examples, including an enzyme such as ß galactosidase and proteins conferring antibiotic resistance or susceptibility. Other examples

include proteins that augment or suppress abnormal proteins, as well as those that are toxic or deleterious to abnormal cells within the body. One example of the latter is the herpes simplex virus thymidine kinase gene. The addition of ganciclovir to cells expressing this gene results in death of the cell. Still others are those which are defective or missing in the patient to be treated, for example, the cystic fibrosis transmembrane regulator gene ("CFTR") can be added to cells containing mutant CFTR with subsequent correction of the ion transport defect caused by the mutant CFTR gene. Examples of other genes currently being investigated for use in gene therapy include adenosine deaminase, insulin, coagulation factors such as factor VIII, and glycogen degrading enzymes.

Although the sequences incorporated into the first and/or second nucleic acids will typically be nucleic acids encoding proteins, the sequences themselves may also be biologically active. Many examples of such materials are known, for example, antisense and ribozymes. Unless specifically stated otherwise, the genes encode therapeutic molecules including biologically active nucleic acids, nucleic acids encoding biologically active proteins, and nucleic acids encoding proteins responsible for producing the biologically active molecules of interest, whether protein or other type of molecule.

For example, for human gene therapy use, one of the retroviral nucleic acid constructs could be modified to contain a "suicide gene" so that the virus producing cells could be eliminated as desired. As one specific example, the neomycin resistance coding sequences could be excised from pBAG, described in detail in the following

examples, with appropriate restriction endonucleases and replaced with herpes simplex virus thymidine kinase coding sequences. It is well established that cells expressing the viral thymidine kinase gene product can be eliminated by treatment with the antiviral agent, ganciclovir (Moolten 1986 Cancer Research **46**:5276). In this manner, the virus producing cells would be eliminated by systemic administration of FDA-approved ganciclovir.

Means for Co-Delivery of First and Second Nucleic Acid Sequences

As described herein, the method for gene therapy can be *in vivo*, i.e., administered directly to a patient for expression of a gene as defined above or for targeted killing of cells, or *in vitro*, for administration directly to cells outside of the body, for expression of exogenous genes. In a preferred example of the latter, cells are obtained from a patient, the first and second nucleic acid sequences administered to the cells, and the cells returned to the patient. An example of this method is the treatment of stem cells or progenitor cells obtained from a patient following cytokine administration to enhance proliferation and mobilization of the stem cells and progenitor cells in the peripheral blood. The genetically engineered cells are then returned to the patient. Alternatively, the cells can be maintained in culture for production of the molecules encoded by the exogenous genes carried by either the first and/or second nucleic acid sequences.

In general, preferred means for co-delivery include the use of (1) a first nucleic acid sequence including infective replication- [illegible]

sequence ionically or covalently coupled to the second nucleic acid sequence, alone or in further combination with an enhancer of transduction, (3) intact infective virus in combination with the first nucleic acid sequences coupled with the second nucleic acid, where the intact virus is rendered non-viable after infection by ultraviolet irradiation in the presence of 8-methoxypsoralen or other free radical initiators such as methylene blue, and (4) delivery of both the first and second nucleic acid sequences separately, by viruses utilizing different receptors.

In the preferred embodiment, the first and second nucleic acid sequences are introduced simultaneously into the cells where virus is to be replicated. The first nucleic acid within a virus is delivered to the cells by the viral infection medium, and the second nucleic acid is added separately by a non-viral means, for example, naked DNA administration or coupled with an enhancer of transduction. The second nucleic acid can also be coupled to the virus containing the first nucleic acid by means of an agent on the viral surface having an affinity for nucleic acids, such as polylysine, followed by the addition of free agent to further condense the second nucleic acid, after which the entire complex is administered to the cells. Alternatively, the second nucleic acid can be coupled to an inactivated virus by means of an agent on the viral surface having an affinity for nucleic acids by first inactivating the virus followed by all the other steps used to make a complex as described above. The first and second nucleic acids can also be coadministered by viruses utilizing different receptor types, a requirement to avoid receptor interference

Infection with the defective retrovirus:

Retroviral-mediated delivery of the sequences required for replication of defective viruses can be accomplished using retroviral vectors or other vectors known to those skilled in the art. For example, E1A sequences can be delivered using the pLN retroviral vectors developed by Miller and colleagues (Miller 1989 BioTechniques **7**:980), provided A.B. Miller, Fred Hutchinson Cancer Research Center, Seattle, WA. The desired E1A sequences are excised with appropriate restriction endonucleases from a parent plasmid construct, blunted and ligated into a similarly blunted cloning site of pLNSX, described by Miller, 1989 BioTechniques **7**:980. Since pLNSX contains an SV40 early promoter upstream of the cloning site, this can be excised by a *Bam* HI/*Hind* III digest at the time of linearizing the vector, after which the sites are be blunted. The method described by Butterworth and Miller (Miller 1986) can be used in which the plasmid retroviral vector containing the E1A sequences is transfected into the Psi2 ecotropic packaging line described by Cepko 1984 Cell **37**:1053, without selection. The transient supernatant containing some ecotropic virus is collected 48 hours later and used to infect the PA317 ecotropic packaging cell line by Miller 1986 Mol. Cell Biol. **6**:2895. Individual producer clones are selected by culturing the cells in G418 media, amplifying and testing for titer by methods described by Rousculp 1992 Human Gene Therapy **5**:471.

Co-delivery of first and second nucleic acid sequences:

Examples of materials facilitating co-

sequences [illegible]

couple the nucleic acid sequences, such as

polylysine, as well as biotin conjugation with streptavidin and other cross-linking agents that can be used with standard technology to covalently couple the first and second nucleic acid sequences.

Coupling by means of an agent ionically binding nucleic acid, such as polylysine, is described by the U.S. patent application entitled "Composition for Introducing Nucleic Acid Complexes into Higher Eucaryotic Cells" filed September 2, 1992 by David T. Curiel, Max L. Birnstiel, Matthew Cotten, Ernst Wagner, Kurt Zatloukal, Christian Plank, and Bernd Oberhauser, the teachings of which are incorporated herein.

DNA can be coupled using a covalent crosslinking agent, for example, polyglutaraldehyde (Digene Diagnostics), or more typically, by biotinylation and linkage via an avidin (streptavidin) bridge to the viral surface. Biotinylation is well known to those skilled in the art, for example, as described by Avignolo, et al., 1990 Biochem. Biophys. Res. Commun. 170:243-250.

Example 1 demonstrates a preferred embodiment for co-delivery of pE1A (a second nucleic acid sequence consisting of a plasmid and the GRR for a replication-defective adenovirus) and AdCMVlacZ (a replication-defective adenovirus including the CMV promoter and lacZ bacterial gene encoding ß galactosidase as a marker gene) accomplished by ionically linking the pE1A to the virus exterior. Figure 2A is a map of the AdCMVlacZ viral genome. Hatched bars represent the conserved human type 5 adenovirus sequences with map units indicated below. CMV promoter sequences ("CMV"), bacterial lac Z sequences ("lac Z"), and SV40 polyadenylation signal ("pA") were inserted as cassette in place of the deleted E1A and E1B sequences. Figure 2B is a map of pE1A. Adenovirus

type 5 nucleic acids are shown ("Ad 5") with nucleotide numbers above and map units below the indicated fragment. The Ad 5 nucleic acids were ligated into the multiple cloning site of pUC 13 represented by hatched bars at the indicated restriction endonuclease sites.

Molecular conjugates can also be used to deliver the E1A nucleic acid sequences. These consist of a means for ionically linking the first and second nucleic acid sequence via a covalently coupled conjugate. One example is the polylysine-conjugated transferrin (TfpL) commercially available from SIGMA Chemical Co., St. Louis, MO (Cat. # T 0288). The optimal proportions of nucleic acid to TfpL are established in preliminary experiments with a reporter plasmid. The E1A sequences are mixed with the TfpL and added to the target cells.

<u>Infection with a virus which is then rendered non-viable</u>

A virus capable of normal infection can be rendered non-viable by exposure to a dye such as 8-methoxypsoralen which kills virus in the presence of ultraviolet radiation, as described in detail by Cotten, et al., 1992 <u>Proc. Natl. Acad. Sci. (USA)</u> **89**:6094, after which the first and/or second nucleic acids can be coupled to the inactivated surface as described above.

Other useful materials include methylene blue and other free radical initiators and dyes that are known to selectively damage viral DNA.

<u>Transduction Enhancers</u>

The first and second nucleic acid sequences may be transduced separately or together into the cells where viral replication is to occur.

[illegible]

[illegible] known [illegible] those skilled in the art, for example, <u>Gene transfer and</u>

expression: a laboratory manual Kriegler M. 242 pp. (W.H. Freeman, NY 1991); and Current Protocols in Molecular Biology, 1987-1994, Ausubel F.M., et al., section entitled "Introduction of DNA into Mammalian Cells" pp. 9.0.1-9.17.2 (John Wiley & Sons).

In a preferred example of a transduction enhancer, cationic lipids, which typically are phospholipids modified to obtain a positive charge, are used for transduction of the first and second nucleic acid sequences. A number of lipid compounds shown to have efficacy for nucleic acid transduction, for example, the cationic lipid N-[1-(2,3-Dioleoyloxy)propyl]-N,N,N-trimethyl-ammoniummethylsulfate (DOTAP), can be used. The stock solution commercially obtained from Boehringer Mannheim is diluted three-fold with HEPES buffered saline and mixed with DNA at 50 ng/μl. The typical ratio of lipid to DNA (weight:weight) is 6:1, but other ratios may provide better results, as determined by empiric testing. After allowing the DNA and lipid to associate for 10 minutes, the lipid-DNA complexes are administered to the target cells.

In each of these alternative delivery methods, the precise conditions for the cotransduction can be easily established by those skilled in the art using the teachings provided here. In certain cell types, optimal results are obtained by separating the infection with the virus from the transduction of the complementing GRR sequences. In addition, some cell types or physiologic situations may produce optimal results with a specific means of GRR nucleic acid delivery. Furthermore, it is possible that multiple transductions of the GRR and/or the [illegible] may result in additional virus production.

Examples

Co-transduction of replication-defective adenovirus

Co-transduction of replication-defective adenovirus with the required nucleic acid sequences for replication was employed in the following examples, demonstrating the successful development of compositions and methods for making recombinant adenovirus from cotransduced cells that prior to transduction did not make any virus or contain any virus nucleic acids. The recombinant adenovirus employed was AdCMVlacZ (Yang 1993 Proc. Natl. Acad. Sci. (USA) 90:4601), an E1A-deleted virus (map units 1.3 - 9.4 deleted) that included a CMV promoter-driven, bacterial lac Z gene. A viral stock of AdCMVlacZ was amplified in 293 cells and modified to contain polylysine molecules on the virus exterior using methods described in detail in Example 1. A plasmid, designated pE1A, was designed to contain the E1A nucleic acids deleted from AdCMVlacZ. The pE1A plasmid contained nucleotides 1- 5768 of the human adenovirus type 5 genome that included E1A and E1B. Although the pE1A plasmid was derived from another adenoviral plasmid, the required nucleic acids could be easily derived from adenoviral infected cells, or directly from adenoviral DNA that does not contain any deletions in the E1A region.

The possibility of recombination can be eliminated, or greatly reduced, by making a complementary plasmid that contains the bare minimum of sequences necessary to complement those missing from AdCMVlacZ, or related E1A-deleted viruses. Although the E1A region encodes proteins of 243 and 289 amino acids (Flint 1989 Ann. Rev. [illegible] [illegible] amino [illegible] trans activating the other viral genes and

establishing lytic infection (Winberg 1984 <u>EMBO Journal</u> **3**:1907; Moran 1986 <u>J. Virology</u> **57**:765). Very recently, exon 2 of the E1A region was shown to be sufficient for transactivation of the other viral genes, although the degree of activation was variable in different cell lines (Mymryk 1993 <u>J. Virology</u> **67**:6922). Therefore, one can utilize smaller portions of the E1A region than employed in the following examples. The variation of trans activation by exon 2 of E1A in different cell types can also be used to target viral replication to cell types in which exon 2 of E1A alone is a sufficient trans activator.

Although one recombinant adenovirus is used in the following examples, it is evident that the approach can be used for trans complementation of other adenoviral genes, and other adenoviral serotypes. For example, fibre-deleted adenoviral mutants and methods for propagating those mutants have been described by Falgout 1987 <u>J. Virology</u> **61**:3759; Falgout 1988 <u>J. Virology</u> **62**:622. In the case of the fibre-deleted mutant, the strategy would employ the co-delivery of the recombinant virus with the deleted fibre gene and a separate nucleic acid encoding the deleted fibre sequences. Since trans complementation of replication-defective adenoviruses using cell lines that contain complementing nucleic acids has also been described for E4 and E2A deletion mutants (Weinberg 1983 <u>Proc. Natl. Acad. Sci. (USA)</u> **80**:5383; Klessig 1984 <u>Mol. Cell. Biol.</u> **4**:1354), these are additional replication-defective adenoviruses that could be complemented in trans using the methods described here. Furthermore, this type of approach could be used to mix genes of different adenoviral serotypes to produce new tropisms or other biological effects.

Co-transduction of replication-defective retrovirus

Recombinant retrovirus was also produced by cells cotransduced with recombinant viral and complementing viral nucleic acid sequences. The recombinant retroviral vector used in the example shown here, pBAG, contains a bacterial lac Z gene and a neomycin resistance gene in place of deleted viral genes gag, pol and env (Price 1987 Proc. Natl. Acad. Sci. (USA) **84**:156). A plasmid vector containing the deleted retroviral genes, pPAM3, was chosen as the complementing nucleic acid because this plasmid had been used to create the widely used packaging cell line, PA317 (Miller 1986). Both the vector and complementing plasmids were co-delivered by ionic linkage to polylysine conjugated to the exterior of the DL1014 adenovirus (Bridge 1989 J. Virology **63**:631) using the methods detailed in Example 2.

Co-transduction of retrovirus and adenovirus genes

The examples described below are specific to either replication-defective adenovirus or to replication-defective retrovirus. An advantage of retrovirus is that it only infects replicating cells, as reported by Miller 1990 Molecular and Cellular Biology **10**(8):4239. This feature has been used to target replicating cells in a therapeutic context, as described by Culver 1992 Science **256**:1550. This is particularly useful in cancer treatment since the tumor cells typically replicate more rapidly than the normal host cells. In the brain, the tumor cells are typically the only replicating cells.

Accordingly, one can use a combination of retrovirus and adenovirus to replicate replication-defective virus selectively in replicating cells. The retrovirus still infect only replicating cells.

It is used as a carrier for the GRR for adenovirus (E1A). The cells are also infected with replication-defective adenovirus. Since the purpose of infecting the cells is to kill the cells, and infection and replication of adenovirus in cells results in cell lysis and death, no additional non-viral genes are required to kill the targeted cells.

In addition to the methods described above, recombinant adenoviruses containing one or both of the complementary retroviral nucleic acid sequences can also be constructed and used. In this case, the replication-defective virus is a retrovirus and the replication-defective adenovirus is a carrier for the GRR for the retrovirus. The methods described by Graham and Prevec (1991) Methods in Molecular Biology. Clifton, The Humana Press Inc. 109, are used to create replication-defective adenoviruses containing the retroviral nucleic acids at the site of the E1A deletion. In brief, the retroviral nucleic acid sequences are ligated into a blunted cloning site of pXCJL-2 plasmid that contains the E1A deletion portion of the virus. Standard techniques are used for the plasmid construction and identification. The recombinant virus is made by co-transfecting the 293 cell line (which provides E1A viral proteins in trans) with the pJM17 vector that is designed to homologously recombine with the pXCJL plasmid derivatives to produce a full length, packagable viral transcript lacking E1A, as described by Graham 1991.

The following non-limiting examples demonstrate actual reduction to practice of the methods and compositions described herein, as well as preferred embodiments.

Example 1. Creation of Syngeneic Recombinant Virus-Producing Cells by Cotransduction of a Replication - Defective Virus and a Replication-Enabling Plasmid

a. <u>Construction of the Replication-Enabling Plasmid</u>

A plasmid was constructed to contain the region of adenoviral genome deleted in a replication-defective adenovirus. The replication-defective, human adenovirus, AdCMVlacZ, was unable to replicate in most cells because the lac Z gene had been inserted within the deleted E1A region of the viral genome, as described by Yang 1993. A second replication-defective human adenovirus also used was AdCMVluc which contains a firefly luciferase gene within the deleted E1A region (Herz 1993 <u>Proc. Natl. Acad. Sci. USA</u> **90**:2812). The maps of AdCMVlacz and AdCMVluc are shown in Figure 2. Therefore, the E1A adenoviral region was isolated and inserted into a plasmid vector.

The E1A region was isolated from the plasmid pEcoRIA containing nucleotides 1-27331 of human type 5 adenovirus (Berkner 1983 <u>Nucleic Acids Research</u> **11**:6003). Although the pEcoRIA plasmid contained the desired E1A region, it was found in the course of experimentation to undergo frequent recombination events in the course of routine plasmid amplification that reduced its usefulness for the desired application. Adenoviral nucleotide sequences 1-5768 were excised from pEcoRIA by restriction endonucleases *Eco* RI and *Bst* 1107 I. The fragment was isolated from the parent nucleic acid by agarose gel electrophoresis and extracted from the gel using the GeneClean™ II Kit (Bio 101, La Jolla, CA). The blunted E1A-containing fragment was ligated to the *Eco* RI/*Hinc* II site of pUC 19 within the multiple cloning site of that vector identity of the final plasmid, designated pE1A (map

shown in Figure 2), was confirmed by restriction endonuclease analysis that compared observed fragment sizes to those predicted by a computer generated restriction endonuclease map of that fragment.

A second replication-enabling plasmid was made to contain a smaller portion of the adenoviral E1A region. The *Eco* RI and *Bst* 1107 I fragment containing adenoviral nucleotide sequences 1-5768 was cleaved with *Nco* I to release a fragment containing adenoviral nucleotide sequences 1-2206. This fragment was blunted and ligated to the blunted *Eco* RI/*Hinc* II site of pUC 13 within the multiple cloning site of that vector. Identity of the plasmid, designated pE2206 (map shown in Figure 2), was confirmed by restriction endonuclease analysis.

b. <u>Production of Adenovirus to be Co-Delivered with Replication-Enabling Plasmid</u>

The AdCMVlacZ virus or AdCMVluc virus was amplified by standard techniques by passage through 293 cells. The 293 cells were propagated by routine tissue culture techniques to form an almost confluent monolayer in 10-20, 175 cm^2 flasks. The cells were inoculated with a stock of the AdCMVlacZ at a multiplicity of infection of approximately 100 to 1000:1 for 2 hrs in a minimal volume of 2% fetal calf serum-containing media. After this period, the tissue culture flasks were supplemented with 10% fetal calf serum-containing media ("regular media") at twice the inoculating volume. Three days later, the infected cells were scraped off the plate and isolated by centrifugation (8000 RPM at 4°C for 30 min in a Beckman JA-17™ rotor). The cell pellet was resuspended in between [illegible] [illegible] regular media, after which the cells were lysed by four consecutive freeze-thaw cycles. The lysate

was clarified by centrifugation (8000 RPM at 4° for 30 min in Beckman JA-17™ rotor) and layered on top of a cesium chloride gradient in Beckman SW-28™ centrifuge tubes. The gradient consisted of 20 ml 1.33 gm/ml cesium chloride in 5 mM HEPES on top of a 10 ml 1.45 gm/ml cesium chloride in 5 mM HEPES cushion. Typically, between 6 and 7 ml of lysate was applied to each tube. The viral particles were concentrated within the gradient by spinning the tubes for 90 min at 20°C at 18,000 RPM. The viral band was extracted by a syringe needle introduced into the side of the tube. The aspirated virus was diluted with an equal volume of 5 mM HEPES, pH 7.8 and applied to the top of a cesium chloride gradient within Beckman SW 41™ tubes. The gradient in these tubes consisted of 3.5 ml 1.33 gm/ml cesium chloride in 5 mM HEPES on top of a 3.5 ml 1.45 gm/ml cesium chloride in 5 mM HEPES cushion. Typically, each tube received between 4 and 4.5 ml of the virus isolated from the first gradient spin. The viral particles were concentrated within the gradient by spinning the tubes for 18 hrs at 20°C at 26,000 RPM. After this spin, there was usually a prominent lower band and a fainter upper band. The lower band was aspirated with a syringe needle as described after the first gradient spin.

As one approach to co-delivering the adenovirus and the pE1A plasmid, the adenovirus was prepared to contain a moiety capable of binding plasmid DNA to its exterior. To this end, polylysine was attached to the exterior of the adenovirus isolated as described below. The aspirated virus was adjusted to a volume of 2.5 ml by the addition of 1.33 gm/ml cesium chloride in 5 Mm HEPES. A PD-10™ column (Pharmacia, Piscataway, [illegible] HEPES [illegible] mM NaCl. [illegible] solution was loaded onto

the PD-10 column by gravity, then eluted with 2 ml of HBS. The column eluate was adjusted to a volume of 3.6 ml by adding HBS. Polylysine solution was made by dissolving 100 mg of polylysine (SIGMA #P-2636) in 10 ml HBS, adjusting Ph to 7.8 with NaOH, followed by adjusting total volume to 16.8 ml by the addition of HBS. 0.4 ml of the final polylysine solution was added to the 3.6 ml of viral eluate. EDC linker solution was made by dissolving 1 gm EDC (Pierce, #22980G) in a total volume of 4 ml distilled water. 40 μl of the final EDC solution was added to the polylysine/virus mixture, rapidly but gently mixed with a pipet, then the mixture incubated on ice for 4 hrs. Following this incubation, 8 ml of cesium chloride in 5 mM HEPES was added, mixed by pipet, and the virus concentrated by centrifugation in an SW 41™ rotor at 25,000 RPM for 18 hrs at 20°C. The viral band is again aspirated by a syringe needle as previously described and diluted with an equal volume of viral preservation media (50 % glycerol, 10 mM Tris pH 8.0, 100 mM NaCl, 1 mg bovine serum albumin/ml). The number of viral particles was estimated by spectroscopy at 260 mM in which 1 O.D. = 1 X 10^{12} viral particles. The virus was stored in aliquots at -70°C until further use.

c. <u>Testing of the Polylysine-Conjugated Adenovirus</u>

It was necessary to test the conjugated adenovirus to establish the optimal number of viral particles and amount of plasmid to effect maximal expression of coding sequences within the plasmid. To do this, a range of viral particle numbers: 0.75 X 10^9, 1 X 10^{10}, and 2.5 X 10^{10} were diluted to a total volume of 250 μl by the addition of viral preservation medium. [illegible] microgram of the constitutively expressed reporter plasmid, pGL2 control (Promega, Madison, WI) was added to a total

volume of 250 μl HBS, and this mixture added to the viral particles. The DNA was allowed to complex with the polylysine conjugated to the adenovirus for 30 min at room temperature, after which 4 μg of free polylysine (stock solution = 1 μg/μl in distilled water) was added to 246 μl of HBS, and the mixture added to the DNA/adenovirus mix. The free polylysine was permitted to further complex with the DNA/adenovirus complex so as to further condense the DNA for an additional 30 min at room temperature. Groups received either 9 or 12 μg of plasmid DNA and either 6 or 8 μg of free polylysine in order to examine whether higher amounts of plasmid per viral particle resulted in better expression of the reporter gene within the plasmid.

The final complexes of adenovirus and DNA were applied to cells that were subsequently tested for reporter gene expression. The cell lines HeLa, A549, or others were typically plated at densities of 1.5-2.0 X 10^5/35 mm culture dish the night before the complexes were added. Each plate received 1/10 of the total complex (i.e., 75 μl) in 1 ml of 2% fetal calf serum. Following a 2 hr incubation under usual culture conditions of temperature, humidity and CO_2, the plates were washed 3 times with phosphate buffered saline and replaced with regular media. Two days later, cell lysates were harvested and analyzed for luciferase activity using the instructions and reagents of the Luciferase Assay System, Promega. It was commonly observed that 2.5 X 10^{10} viral particles with 6 μg of plasmid DNA produced the optimal expression of the reporter gene.

d. <u>Demonstration of New Adenovirus by Cells Infected with Adenovirus and Simultaneously Transduced with the Replication-Enabling Plasmid</u>

Experimentation established that cells infected with an E1A-defective virus and simultaneously transduced with an E1A-containing plasmid in trans resulted in the production of new virus by the infected cells. The pE1A plasmid was complexed to the exterior of the polylysine-conjugated AdCMVlacZ using the optimized viral particle numbers, plasmid amounts, and methods as described above. As a control in these experiments, the polylysine-conjugated AdCMVlacZ was complexed with the pUC 13 plasmid that did not contain any adenoviral sequences. The complexes were applied to the human prostate adenocarcinoma cell line, PC-3, that had been plated at a density of 1.5-2.0 X 10^5 cells/35 mm plate. Each plate received 1/10 of a complex in quadruplicate under conditions described in the previous section. Two hrs later, the plates were washed thrice with phosphate buffered saline and regular medium applied. Two, three and four days later, supernatants and cell lysates were collected. The supernatants were prepared by aspirating the media from the plates for each group, clarifying by centrifugation (1500 RPM at 4°c for 5 min) and 0.45 μM filtration, after which the media was stored at -70°C. The lysates were prepared by scraping the plates, then pooling the cells from each group in a total volume of between 0.5 and 0.7 ml regular media. The cells were exposed to four freeze-thaw cycles, the supernatant clarified by centrifugation (3000 RPM in microfuge for 5 min. at 4°C) and frozen at -70°C.

[illegible] supernatants and lysates were tested [illegible] the presence of new adenovirus by exposure to

293 cells. If new replication-defective virus was present, the 293 cells should develop the classic cytopathic effect (CPE) of rounding and detachment from the culture dish surface. Figure 3 is a graphic representation of the observed cytopathic effects produced by lysates or supernatants from cells cotransduced with AdCMVlacZ and plasmid DNA. The pUC 13 groups were observed up to 96 hrs after supernatant or lysate addition without any cytopathic effect. The lysates and supernatants from the cells that had been exposed to the AdCMVlacZ + pE1A all produced CPE in the 293 cells, although the onset of CPE was faster with the lysates than the supernatants at all harvest times. In contrast, none of the lysates or supernatants from the cells exposed to the AdCMVlacZ + pUC 13 developed any signs of CPE.

Similar experiments using the human ovarian carcinoma cell line, SK-OV-3, obtained from ATCC, also yielded only supernatants and lysates from the pE1A groups that produced CPE in 293 cells.

Experimentation with the AdCMVluc virus shows that new replication-defective virus was by codelivery of virus and a replication-enabling plasmid (Goldsmith 1994 Human Gene Ther. **5**: 1341). Table 1 shows the viral titers measured in the supernatants of cells cotransduced with the AdCMVluc virus and pE1A or pUC13 (control plasmid not containing any replication-enabling sequences). The pE1A plasmid, or the pE2206 plasmid, was complexed to the exterior of the polylysine-conjugated AdCMVluc using the optimized viral paral particle numbers, plasmid amounts and methods as described above. The same controls and [illegible] were used to appl[illegible] [illegible] plasmid complexes to the [illegible] cells.

TABLE 1: TITERS IN COTRANSDUCED CELL SUPERNATANTS

	A[a]		B[1]		C[1]	
Harvest time[b]	pUC	pE1A	pUC	pE1A	pUC	pE1A
72	1.5×10^2	1.3×10^6	-	1.0×10^6	2.5×10^2	2.3×10^6
96	10.0×10^2	1.7×10^6	-	4.0×10^6	7.0×10^2	4.2×10^6
120	21.5×10^2	2.5×10^6	-	8.3×10^6	9.5×10^2	2.6×10^6

a Letters denote separate experiments.

b Hours after cotransduction that supernatants were harvested.

Adenoviral particles present in the supernatants of the PC-3 cells cotransduced with adenovirus and pE1A were quantified by titer assays utilizing 293 cells. 293 cells were plated in 35 mm plates to produce 50-60% confluence the next day. Duplicate dilutions of supernatant from the cotransduced PC-3 cells were added to the 293 cells for 4 hrs, followed by aspiration and a wash with PBS, after which complete media was added. The presence of wild type virus was assessed by applying undiluted 10^{-1}, and 10^{-2} dilutions to HeLa cells under identical conditions to those used for the 293 cell titers. Twenty-four hours later, the media was replaced by molten agar overlay (DMEM/F12 supplemented with 0.65% noble agar, 2% FCS, 25 mM $MgCl_2$ antibiotics). The titer plates were fed by the addition of equivalent amounts of agar overlay every 3 days. Plaques were manually counted independently by two observers seven days later. The supernatants from cells cotransduced with the E1A plasmid and AdCMV-luc had concentration dependent numbers of plaques quantified by limiting dilution to derive the figures of 1 to 8 x 10^2. These results showed that the replication-enabling plasmid caused at least a 4 log increase in new recombinant virus production compared to the controls that received only the pUC 13 plasmid.

Small amounts of presumed wild type recombinant virus has been detected in a minority of the experiments. In one of eight experiments, plaques were detected in HeLa cells exposed to supernatants from pE1A-cotransduced cells (72 hr: 1.1 x 10^1, 96 hr: 7.9 x 10^1, 120 hr: 1.5 x 10^1) and no plaques from the pUC-cotransduced supernatants. One other experiment looking only at the 72 hr time [illegible] plaques per ml. However use of the [illegible] replication-enabling plasmid has not

resulted in any wild type plaque formation in the HeLa cells in seven out of seven experiments.

The new virus made by the cells cotransduced with the pE1A and AdCMVluc virus was shown to be functionally similar to the starting virus based on luciferase transfer assays (Goldsmith 1994 Human Gene Ther. 5:1341). Table 2 shows the luciferase transfer capacity of supernatants from PC-3 cells cotransduced with the AdCMVluc virus and pE1A or pUC13. Since the AdCMVluc virus contains the capacity to transfer the functional luciferase gene to new cells with subsequent luciferase production, it would be expected that the if the new virus present in the supernatant of the cotransduced cells is AdCMVluc, it should also transfer luciferase activity to new cells. Supernatants were aspirated from cotransduced cells at the times indicated in the text, clarified by centrifugation and stored at -70°C until testing. Lysates from the cotransduced cells were made by pooling all the enzymatically detached cells from each group (4 plates) in 1.2 ml complete media, freeze-thawing four times, clarifying the lysate by centrifugation and storing at -70°C. Fresh PC-3 cells were plated (1 X 10^5 cells/well in 24 well plates) the evening prior to lysate or supernatant exposure. The cells were exposed to 50 μl of lysate or 500 μl supernatant for four hours in 0.5 ml low serum media, after which complete media was added. The cells were grown under the conditions described above for 72 hours, and luciferase activity measured in the lysates using reagents and instructions of a kit (Luciferase Assay System, Promega, Madison, WI). Protein concentration was measured in the lysate from each individual well [illegible] the Bradford method using the reagents

and instructions of a kit (Biorad Laboratories, Melville, NY).

TABLE 2: Luciferase Activity in Cells Treated with Lysates or Supernatants from PC-3 Cotransduced with AdCMV-luc and Indicated Plasmids

Lysates

Harvest[b] time	A[a]		B[a]		Ca	
	pUC	pE1A	pUC	pE1A	pUC	pE1A
72	19[c](2.5)[d]	8,000(254)	20(0.9)	5,342(291)	6(0.9)	3,501(167)
96	7 (0.6)	3,104(327)	10(3.8)	4,789(1,615)	8(3.0)	6,910(4,191)
120	9 (0.5)	4,534(385)	11(1.4)	3,984(868)	5(1.0)	2,323(398)

Supernatants

Harvest[b] time	A		B		C	
	pUC	pE1A	pUC	pE1A	pUC	ppE1A
72	12(8)	11,438(1525)	15(8.5)	1,297(120)	210(103)	6,039(335)
96	176(50)	7,472(376)	11(1.2)	7,091(852)	91 (42)	8,582(1,118)
20	163(49)	9,245(565)	85(22)	4,594(389)	6 (3)	2,676(700)

a Letters denote separate experiments.
b Hours after cotransduction that lysate or supernatant were harvested.
c All unbracketed numbers indicate mean RLU's/μg protein.
d All bracketed numbers indicate $\pm$ SEM.

PC-3 cells that had been exposed to lysates and supernatants of the pE1A cotransduced cells had significant levels of luciferase activity detected 72 hours later. The supernatants from all three experiments consistently had greater luciferase-transduction capacity than the lysate from those cells. PC-3 cells exposed to either the lysates or the supernatants of the pUC cotransduced cells had barely detectable levels of luciferase activity in all three experiments. Similar analyses have also *been performed with human cell lines SK-Lu1, SK-OV3, A549 and HeLa that had markedly increased amounts of luciferase transfer activity in groups cotransduced with AdCMVluc and pE1A or pE2206 compared to the controls cotransduced with virus and pUC13. Table 3 shows the luciferase transfer capacity in the supernatants of multiple cell lines cotransduced with the AdCMVluc virus and pE1A pr pUC 13. These additional experiments demonstrated that the replication-enablement could work in multiple cell types, not simply the PC-3 cells.

TABLE 3: Evidence of new Ad CMV luc virus production by assessment of luciferase transfer capacity in supernatants of multiple cell lines exposed to Ad CMV luc and pE2206.

Cell Line	Control[1] RLU's	pE2206 RLU's
HeLa	24.3	934.3
SKlu1	49.7	1301.3
A549	17.7	1310.3
SKOV3	10.3	289.0

As further evidence that new recombinant virus had been made, adenovirus was recovered from the AdCMVlacZ + pE1A-treated cells and shown to be identical to the starting virus by DNA analysis.

[illegible]

centrifugation and pooled from the [illegible] cells that developed CPE following lysate or supernatant

exposure (from the virus + pE1A-treated groups). The supernatant pool was applied to confluent plates of fresh 293 cells; each 10 cm diameter dish received 0.5 ml of supernatant in 4 ml of 2% fetal calf serum media. The remainder of the adenovirus amplification was performed as described above. Following the second cesium chloride gradient, the viral band was aspirated and exhaustively dialyzed against 10 mM Tris pH 8.0 at 4°C. The virus was added to an equal volume of 20 mM Tris pH 7.8, 10 mM EDTA pH 8.0, 1.0% SDS containing proteinase K at 100 μg/ml, then incubated at 37°C for between 2 and 16 hrs. The mixture was extracted once with an equal volume of buffered phenol, and DNA precipitated from the remaining solution with ethanol and sodium acetate. The same steps were repeated using the AdCMVlacZ viral stock in order to provide the parent adenoviral DNA specimen for comparison. The DNA pellets were resuspended in 10 mM Tris pH 8.0, 1 mM EDTA and concentration determined by UV spectroscopy (A_{260}/A_{280}). Aliquots (2 μg) of each specimen were cut with restriction endonucleases *Xho* I and *Sal* I, the fragments size fractionated on 1% agarose and the resulting bands compared for relative size. An ethidium bromide-stained photograph of the gel showed identical fragments were produced by the two viral DNA specimens: adenoviral DNA obtained from PC-3 cells following cotransduction with AdCMVlacZ and pE1A and adenovirus made by directly amplifying AdCMVlacZ viral stock. Specifically, the *Xho*I digest produced visualized fragments of approximately 1.4, 2.4, 2.5, 4.3, 5.0, and 15-20 kb; and the *Sal*I digest produced fragments of approximately 25 and 7 kb. This analysis showed [illegible] AdCMVlacZ [illegible] and that produced by the infection of [illegible] cells in the

presence of pE1A, yielded similar fragments demonstrating that the cotransduction of pE1A and AdCMVlacZ resulted in the production of new, replication defective adenovirus, AdCMVlacZ.

A similar analysis of the virus made by cells cotransduced with AdCMVluc and pE1A also showed the new virus was identical to the starting AdCMVluc virus, as reported in Goldsmith 1994 Human Gene Ther. 5:1341.

e. <u>Demonstration of New Adenovirus Production by Cells Cotransduced with Viral and Plasmid DNAs</u>

Experimentation established that cells cotransduced with both replication-enabling plasmid and recombinant adenoviral DNA led to the production of recombinant adenovirus. This approach has utility in that it is possible to use viral-free methods for the initial introduction of the nucleic acids into cells which subsequently can locally produce the recombinant adenovirus *in vivo*. Viral free systems may have advantages with respect to cost and safety over viral systems, but suffer from lower efficiency of gene transfer. The ability to use viral-free approaches to administer the viral nucleic acids so that recombinant virus could be locally made *in vivo* capitalizes on the advantages of both viral-free and viral gene transfer systems.

AdCMVluc viral DNA was prepared as described previously. The AdCMVluc viral DNA and pE1A plasmid DNA were cotransduced into HeLa cells using cationic lipid, 1,2-dimyristoyl-3-trimethylammonium-propane (DOTAP), as a facilitator of DNA transfection. Experimentation has established that the molar ratio of replication-enabling plasmid [illegible] variable as is the ratio of DOTAP to total DNA being introduced to the cells. These experiments

have shown that a molar ratio (plasmid:viral) of approximately 1:1 to 4:1 is necessary in conjunction with a lipid to DNA ratio of 2:1. Unlike the original method of codelivery (using plasmid ionically linked to the virus exterior) where new virus production was evident at 72 hrs, no new virus could be detected until 6-8 days post-transduction of the DNAs.

The plasmid and viral DNAs were gently mixed together in OPTIMUM® (GIBCO/BRL) to produce a total DNA concentration of 100 ng/μl. In a separate tube, DOTAP was mixed with OPTIMUM® to yield a lipid concentration of 200 ng/μl. The DNA tube contents were then added and gently mixed with the DOTAP tube, and the tube incubated at room temperature for 10 minutes. HeLa cells (10^5 cells/well of a 24 well plate) were washed, and 200 μl of OPTIMUM® (GIBCO/BRL) added. After the DOTAP/DNA incubation period, 10 μl of the mixture was added to each well of the HeLa's that were subsequently placed in the usual 37OC humidified/CO_2 atmosphere. Four hrs later, the media was aspirated and replaced with DMEM/F12 supplemented with 2% fetal calf serum media. Supernatants were collected 2, 4, 6, and 8 days later and examined for the capacity to transfer luciferase activity to fresh HeLa cells as evidence of new AdCMVluc production. Luciferase activity was only conferred by supernatants from the cells 6-8 days post-transduction as graphically depicted in Figure 4.

f. <u>Demonstration that Replication-Enablement Increases Gene Transfer in Tumor Tissue *in vivo*</u>

Experiments suggest that the replication-enablement technology amplifies adenoviral-[illegible] within a prostate carcinoma model system. [illegible] system was tested by measuring the amount of adenoviral transgene

expression, i.e., luciferase activity, within tumors engrafted in nude mice. Tumors were produced by administering the PC-3 cells (1 x 10^7 cells SQ, flanks) that achieved an 8-10 mm diameter in approximately four weeks in athymic nude mice. At that time, each tumor was injected once with 25 μl of AdCMVluc/pE1A complex made as previously described using a 27 ga. needle, so that each tumor received 8.3 x 10^8 viral particles complexed with 198 ng plasmid DNA. The tumors were excised 10 days later, and lysates made with a polytron on ice, from which luciferase activities and total protein concentrations were measured as described above. In the tumors that were evaluated, this analysis showed that those cotransduced with AdCMV-luc and pUC 13 (n=7) had significantly lower luciferase activity per mg of protein compared to tumors cotransduced with the same virus and pE1A (n=8) (Figure 3). Other experiments have shown a consistently increased amount of luciferase expression in the tumors that received the virus and the replication-enabling plasmid compared to controls receiving virus and pUC 13 as graphically depicted in Figure 5, although significant variability is present within the groups. These results show that replication-enablement of the AdCMV-luc virus within a tumor mass led to greater transfer of the adenoviral transgene.

Example 2. Creation of Syngeneic Recombinant Virus-Producing Cells by Cotransduction of Trans-Complementing Plasmid Constructs.

Recombinant retrovirus is conventionally made by passaging a retroviral vector through a "packaging cell line" that has been stably transfected with complementing viral protein coding [illegible] sequences of the vector and the packaging sequences are designed so that only the

recombinant viral genome will be packaged into new viral particles. The result is an infectious particle that cannot replicate new virus. One widely used retroviral packaging cell line is PA317 that was made by transfecting mouse fibroblasts with the pPAM3 plasmid (Miller 1986).

The appropriate plasmids were cotransduced as follows in order to demonstrate that a plasmid containing the genes required for replication obtained from a packaging cell line and retroviral vector could be co-delivered with the resulting production of new recombinant retrovirus. The pPAM3 plasmid is described by Miller 1986, as noted above. A representative retroviral vector, pBAG, that contained a neomycin resistance gene as a selectable marker and a bacterial lac Z gene as described by Price 1987, was obtained from C.Cepko (Harvard University, Cambridge, MA). Figure 6A is a map of pBAG. Figure 6B is a schematic of retroviral sequences within the pPAM3 plasmid.

The plasmids were introduced into cells by ionic attachment to the exterior of an adenovirus. The human type 5 adenovirus, DL1014 (Bridge 1989), was modified to contain polylysine on the exterior using the techniques described in Example 1. The pPAM3 and pBAG plasmids were mixed in varying proportions: 1 μg pBAG + 5 μg pPAM3, 3 μg pBAG + 3 μg pPAM3, 5 μg pBAG + 1 μg pPAM3, and 6 μg pBAG alone, in a total volume of 250 μl HBS and added to 2.5 X 10^{10} polylysine-conjugated viral particles. After incubation at room temperature for 30 min, free polylysine was added as described in Example 1. A549 cells or PC-3 cells, both at densities of 1.5-2.0 X 10^5/35 mm plate, received 1/10 of each complex in triplicate to quadruplicate for 2 hrs after which the media was changed to regular medium and the cells grown under usual conditions. Three

days post-transduction with the two plasmids, supernatant was aspirated from the cells, clarified by centrifugation followed by 0.45 μM filtration and frozen at -70°C until further testing.

The presence of recombinant retrovirus in the supernatant was demonstrated by standard titer assays. Briefly, the supernatant was diluted by log 10 in regular media supplemented with polybrene at 5 μg/ml. The diluted supernatant was applied to A549 cells (5 X 10^5 cells/60 mm plate) overnight, after which the media was changed to regular media. One day following the media change, the A549 cells were enzymatically detached with trypsin and 1/10 of the cells plated in a fresh culture dish in media supplemented with G418 1 mg/ml. Only cells containing the neomycin resistance gene supplied by the recombinant retrovirus can survive in the G418 media. The result after 10 to 14 days was the appearance of G418-resistant colonies of cells, each colony representing the progeny of a single resistant cell. Figure 5 is a graphic representation of the titers of retrovirus in supernatants of cells cotransduced with pBAG and pPAM3. Supernatants were collected 72 hrs post-transduction with the complementary retroviral vectors. In the supernatant obtained from the cotransduced A549 cells, titers were highest in the groups that received 1 μg pBAG + 5 μg pPAM3 at 4 X 10^2/ml. A similar pattern was observed in PC-3 cells that were cotransduced with varying proportions of the complementing plasmids, resulting in titers of 1 X 10^3/ml.

As further evidence that new recombinant retrovirus had been made, resistant colonies were shown to express the lac Z gene product. The colonies were washed thrice with phosphate buffered saline, fixed 10 minutes at 4°C in 0.2%

glutaraldehyde, 50 mM sodium phosphate buffer pH 7.3. Following the fixation period, the plates were washed thrice with phosphate buffered saline, then incubated with the lac Z protein substrate, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) in "X-Gal Solution" (80 mM Na_2HPO_4, 20 mM NaH_2PO_4, 1.3 mM $MgCl_2$, 1 mg/ml X-Gal, 3 mM $K_3Fe(CN)_6$, 3 mM $K_4Fe(CN)_6$) at 37°C overnight. Cells containing the lac Z protein turn blue after this treatment. Most of the colonies were stained deeply blue, indicating the presence of the lac Z product provided by the recombinant retrovirus.

We claim:

1. A method for creating recombinant virus-producing cells comprising

selecting a first nucleic acid sequence comprising a viral genome which is not capable of directing the production of new viral particles in the absence of additional viral nucleic acid sequence,

constructing a second nucleic acid sequence comprising a viral gene sequence consisting essentially of the viral genes required for the first nucleic acid sequence to produce new viral particles in a cell in which the first nucleic acid sequence is otherwise unable to direct the production of new viral particles, in combination with means for replicating the second nucleic acid sequence, and

inserting the combination of the first and second nucleic acid sequences into the cell in which the first nucleic acid sequence is unable to direct the production of new viral particles in the absence of the second nucleic acid sequence.

2. The method of claim 1 wherein the first and second nucleic acid sequences are coupled.

3. The method of claim 2 wherein the sequences are coupled covalently.

4. The method of claim 2 wherein the sequences are coupled ionically.

5. The method of claim 1 wherein the first and second nucleic acid sequences are inserted by transduction.

6. The method of claim 1 wherein the first and second nucleic acid sequences are inserted by means of a virus capable of infecting the cell.

7. The method of claim 6 wherein the [illegible] [illegible] [illegible] prior [illegible] infection.

8. The method of claim 1 wherein the first or second nucleic acid sequences further comprise nucleic acid sequence selected from the group consisting of nucleic acid sequence that codes for proteins used to identify cells infected with recombinant virus produced by the combination of the first and second nucleic acid sequences, nucleic acid sequences that codes for proteins that function to kill cells containing the viral genome, nucleic acid sequence that codes for therapeutic proteins that will serve to treat a pathophysiologic condition within the body, and nucleic acid sequence encoding biologically active nucleic acids.

9. The method of claim 8 wherein the first nucleic acid sequences comprising coding nucleic acid sequence.

10. The method of claim 1 wherein the virus is selected from the group consisting of adenoviruses, herpesviruses, adeno-associated viruses, and retroviruses.

11. A composition for creating recombinant virus-producing cells comprising

a first nucleic acid sequence comprising a viral genome which is not capable of directing the production of new viral particles and

a second nucleic acid sequence comprising

a viral gene sequence consisting essentially of the genes required for the first nucleic acid sequence to direct the production of new viral particles in a cell in which the first nucleic acid sequence is otherwise unable to direct the production of new viral particles

in combination with means for replicating the second nucleic acid sequence.

12. The composition of claim 11 further comprising a cell in which the first nucleic acid

sequence is unable to direct the production of new viral particles in the absence of the combination of the first and second nucleic acid sequences.

13. The composition of claim 11 wherein the first and second nucleic acid sequences are coupled.

14. The composition of claim 13 wherein the sequences are coupled covalently.

15. The composition of claim 13 wherein the sequences are coupled ionically.

16. The composition of claim 11 further comprising an agent facilitating transduction of a mammalian cell by the first and second nucleic acid sequences.

17. The composition of claim 11 wherein the first or second nucleic acid sequences further comprise nucleic acid sequence selected from the group consisting of nucleic acid sequence that codes for proteins used to identify cells infected with recombinant virus produced by the combination of the first and second nucleic acid sequences, nucleic acid sequences that codes for proteins that function to kill cells containing the viral genome, nucleic acid sequence that codes for therapeutic proteins that will serve to treat a pathophysiologic condition within the body, and nucleic acid sequence encoding biologically active nucleic acids.

18. A method for expressing exogenous nucleic acid sequence in a mammalian cell comprising introducing into a cell

a first nucleic acid sequence comprising a viral genome which is not capable of directing the production of new viral particles and

a second nucleic acid sequence comprising

a viral gene sequence consisting essentially of the genes required for the first nucleic acid sequence to direct the production of new viral particles in a cell in which the first nucleic acid sequence is unable to otherwise direct the production of new viral particles

in combination with means for replicating the second nucleic acid sequence in a cell,

wherein the first or second nucleic acid sequence is selected from the group of nucleic acid sequence consisting of nucleic acid sequence that codes for proteins used to identify cells infected with recombinant virus produced by the combination of the first and second nucleic acid sequences, nucleic acid sequences that codes for proteins that function to kill cells containing the viral genome, nucleic acid sequence that codes for therapeutic proteins that will serve to treat a pathophysiologic condition within the body, and nucleic acid sequence encoding biologically active nucleic acids.

19. The method of claim 18 wherein the first and second nucleic acid sequences are coupled.

20. The method of claim 19 wherein the sequences are coupled covalently.

21. The method of claim 19 wherein the sequences are coupled ionically.

23. The method of claim 18 wherein the cells are in a patient.

24. A method for killing replicating cells comprising

administering to the replicating cells an adenovirus which is unable to direct the production of new viral particles in the replicating cells,

administering to the replicating cells a retrovirus comprising

a viral gene sequence consisting essentially of the genes required for the adenovirus to direct the production of new viral particles in the replicating cells in which the adenovirus is otherwise unable to direct the production of new viral particles.

25. The method of claim 24 wherein the replicating cells are tumor cells.

26. The method of claim 25 wherein the replicating cells are in the brain.

27. A composition for killing replicating cells comprising

an adenovirus which is unable to direct the production of new viral particles in the replicating cells, and

a retrovirus comprising

a viral gene sequence consisting essentially of the genes required for the adenovirus to direct the production of new viral particles in the replicating cells in which the adenovirus is otherwise unable to direct the production of new viral particles.

28. The composition of claim 27 wherein the replicating cells are tumor cells, further comprising the cells.

FIG. 1a

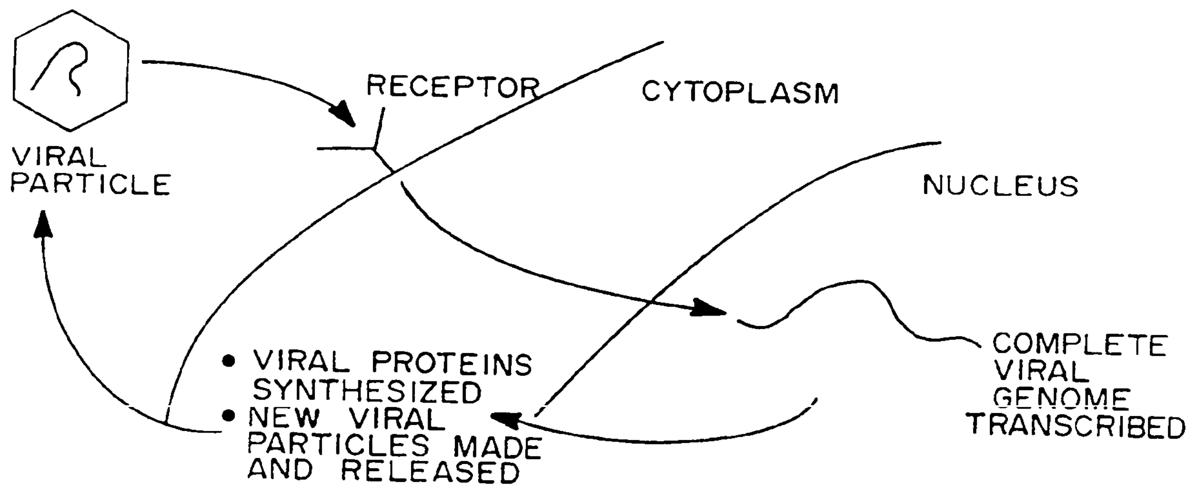
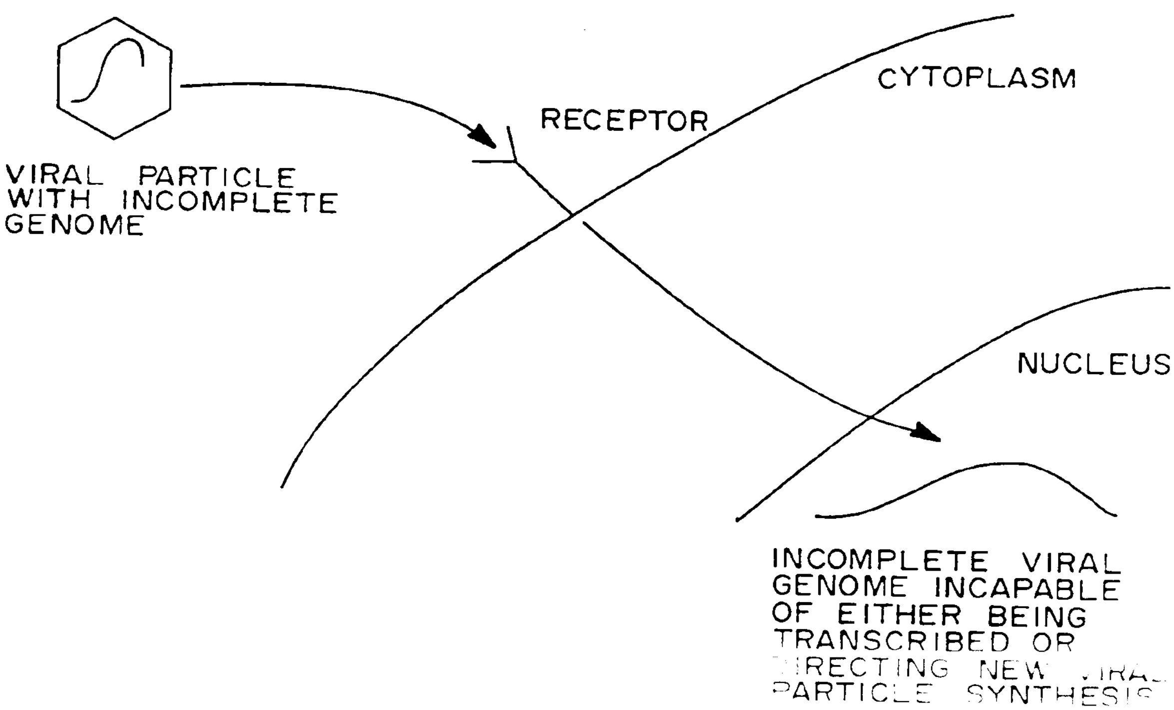

RECEPTOR
CYTOPLASM
VIRAL PARTICLE
NUCLEUS
COMPLETE VIRAL GENOME TRANSCRIBED
• VIRAL PROTEINS SYNTHESIZED
• NEW VIRAL PARTICLES MADE AND RELEASED

FIG. 1b


CYTOPLASM
RECEPTOR
VIRAL PARTICLE WITH INCOMPLETE GENOME
NUCLEUS
INCOMPLETE VIRAL GENOME INCAPABLE OF EITHER BEING TRANSCRIBED OR

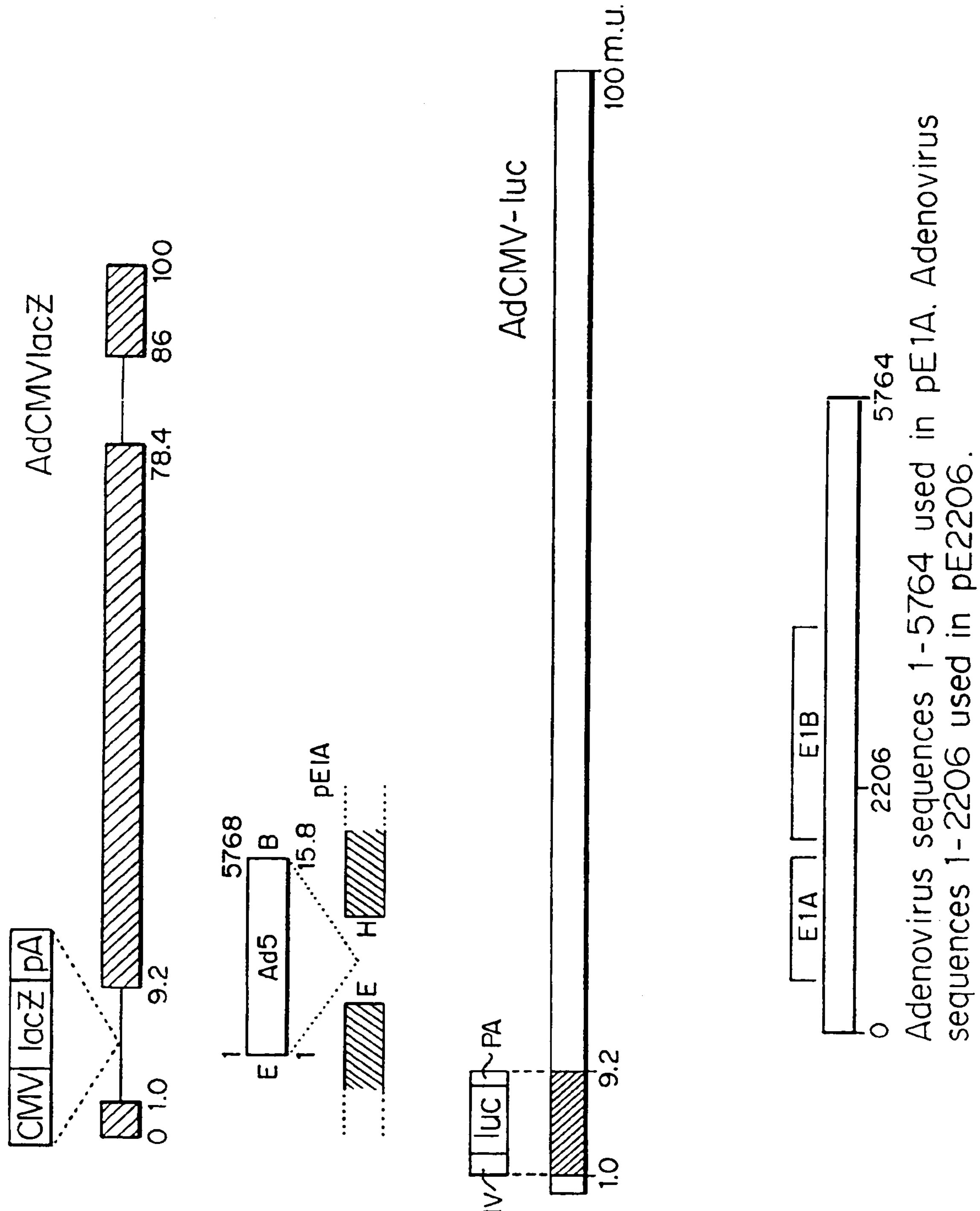


FIG. 2a

FIG. 2b

FIG. 2c

FIG. 2d

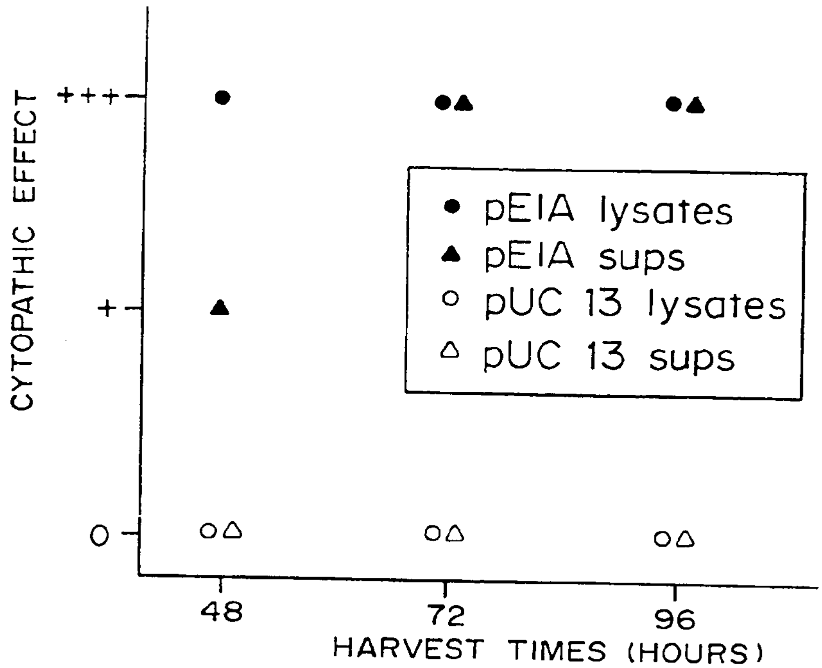

CYTOPATHIC EFFECT
+++
+
0
pEIA lysates
pEIA sups
pUC 13 lysates
pUC 13 sups
48
72
96
HARVEST TIMES (HOURS)

FIG. 3

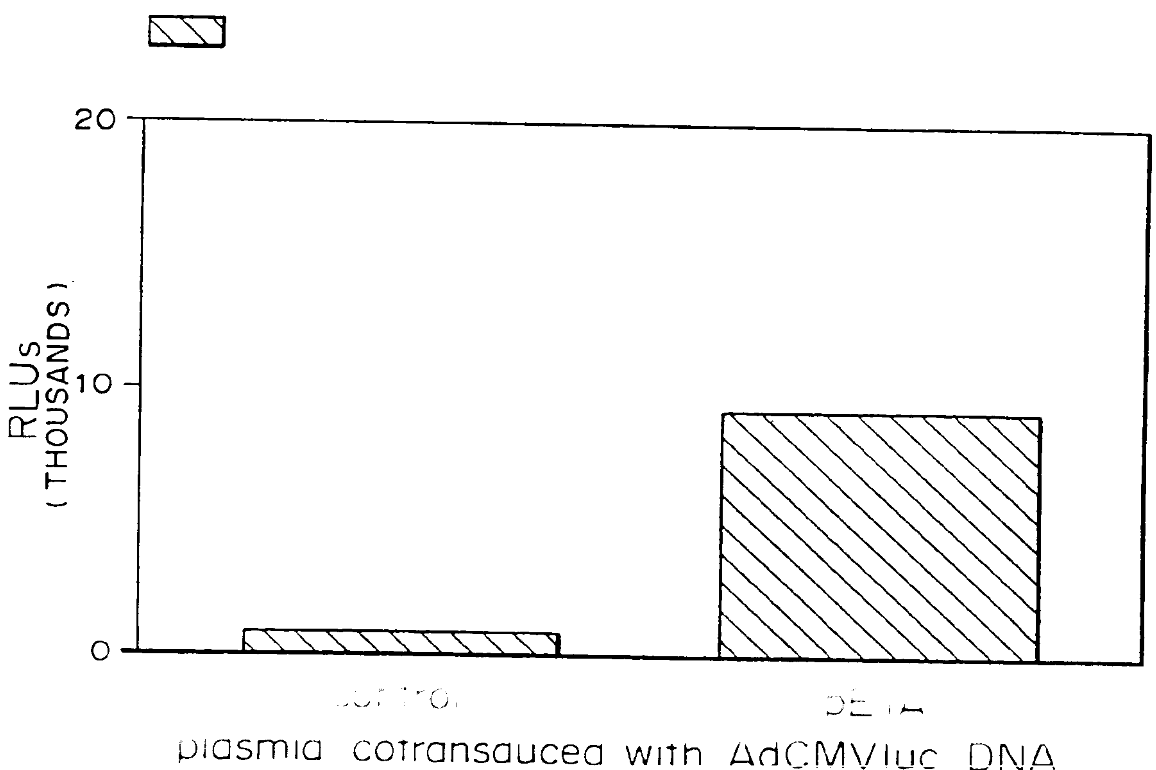

RLUs (THOUSANDS)
20
10
0
plasmid cotransduced with AdCMVluc DNA

FIG. 4

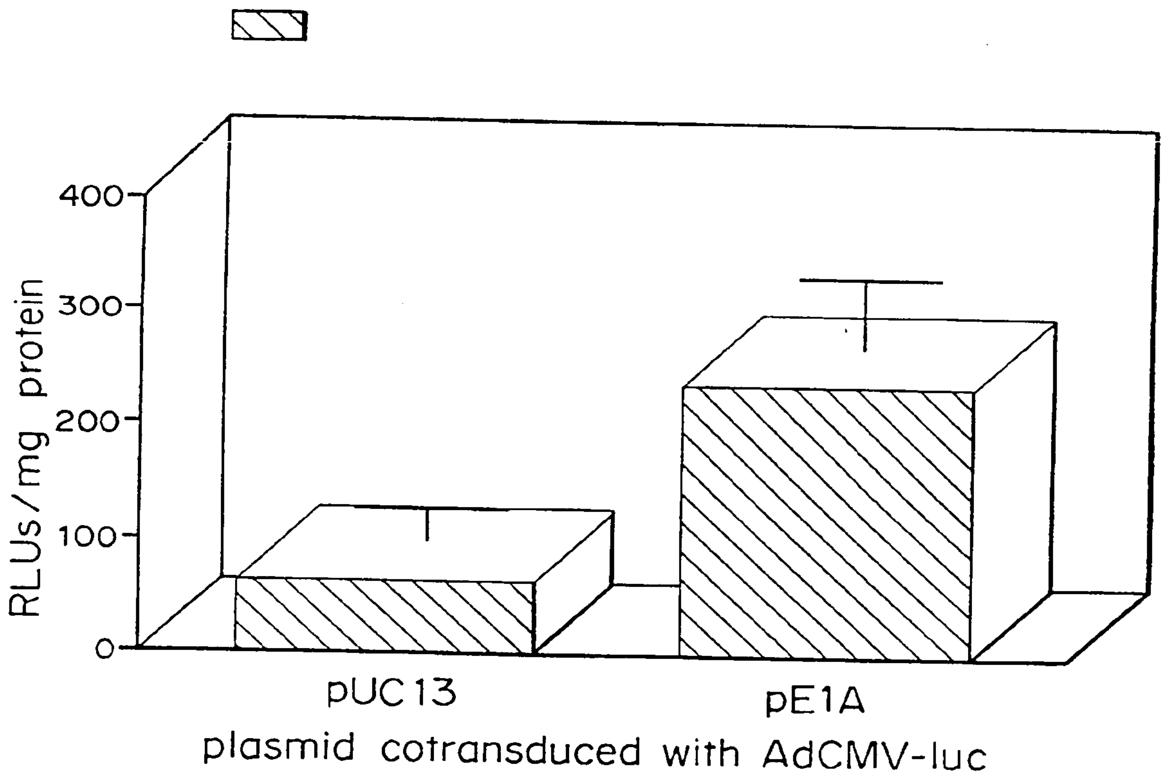

RLUs/mg protein
400
300
200
100
0
pUC13
pE1A
plasmid cotransduced with AdCMV-luc

FIG. 5

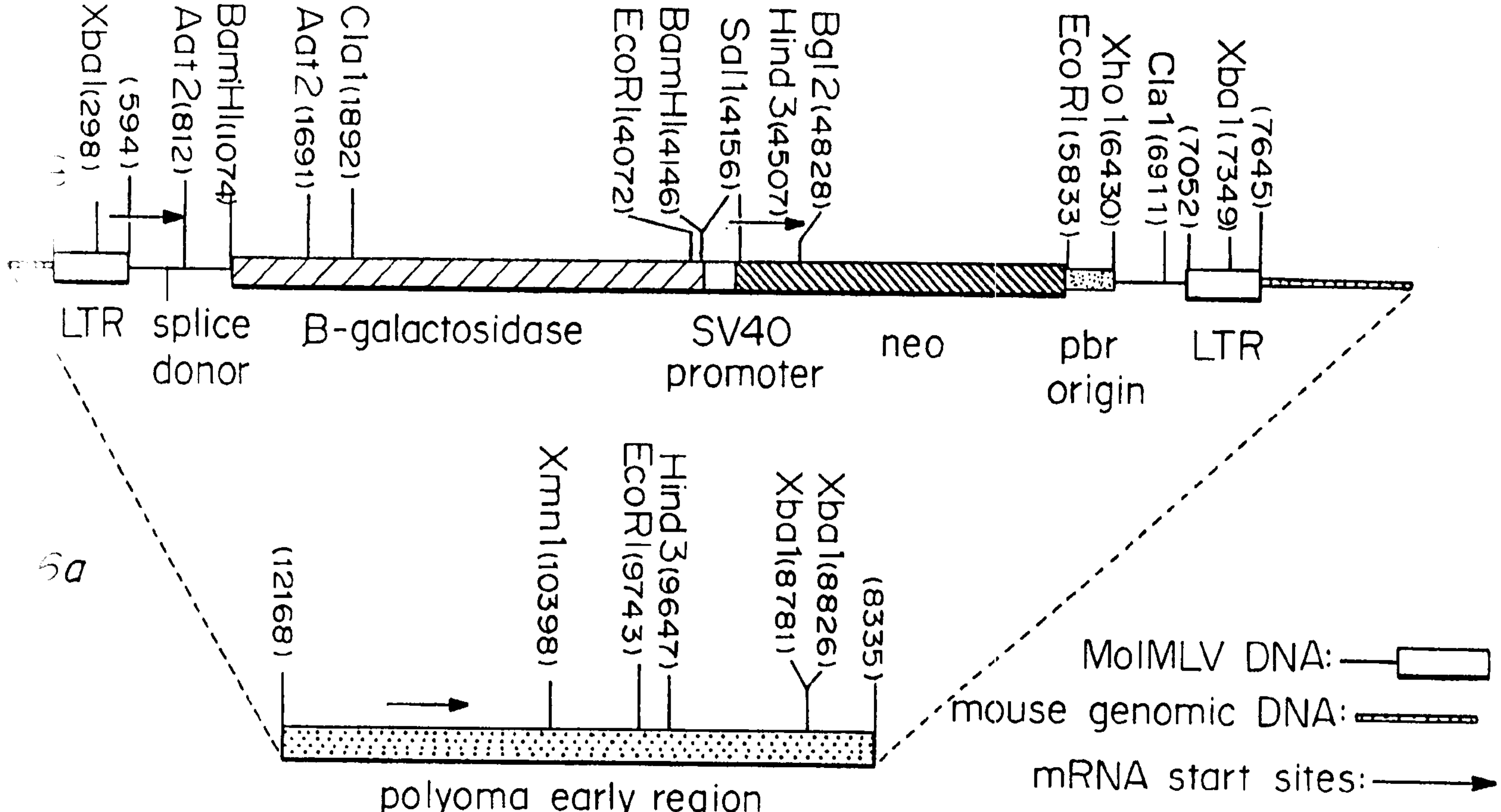


FIG 6a

TOTAL PLASMID SIZE- 12,220bp

NOTES 1) THERE IS NO AMP GENE IN THIS PLASMID.
THE NEO GENE CAN BE SELECTED IN BACTERIA BY GROWTH IN 50 μg/ml KANAMYCIN SULFATE.
2) THE ATG FOR THE β-gal GENE (ORIGINALLY THE ATG FOR THE VIRAL pr65 gag PROTEIN) IS LOCATED 5' TO THE BamHI SITE AT 1074:

met gly ala asp pro val
...ATG GGC GCG GAT CCC GTC...

BamHI(1074)

9TH AMINO ACID OF WILD TYPE β-gal GENE

3) ALL RECOGNITION SITES FOR THE LISTED RESTRICTION ENZYMES ARE SHOWN.
4) REFERENCE: PNAS 84:156-160, 1987

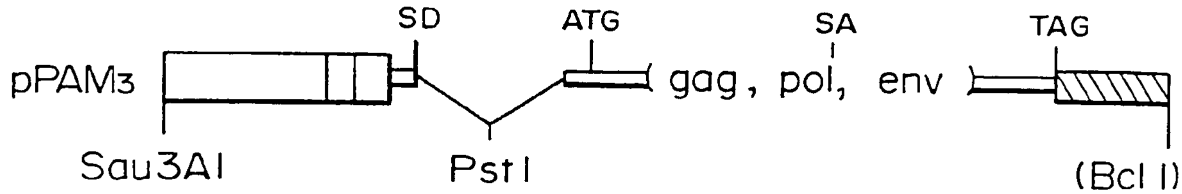

SD
ATG
SA
TAG
pPAM3
gag, pol, env
Sau3AI
PstI
(BclI)

FIG. 6b

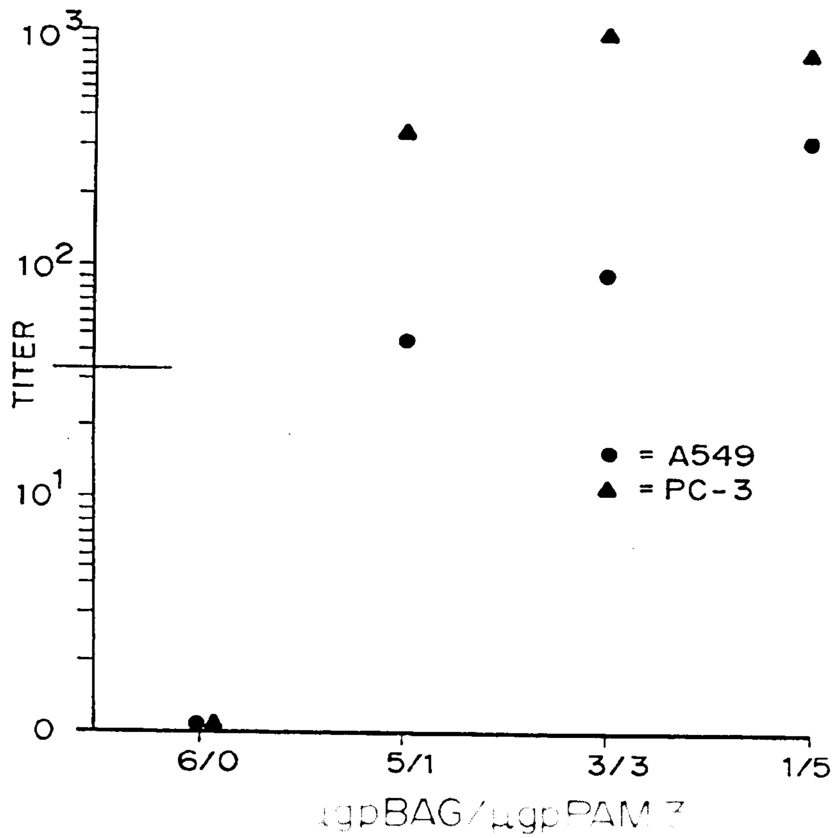

10^3
10^2
10^1
0
TITER
● = A549
▲ = PC-3
6/0
5/1
3/3
1/5

FIG. 7

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(74) Agent: PABST, Patrea, L.; Arnall Golden & Gregory, 2800 One Atlantic Center, 1201 West Peachtree Street, Atlanta, GA 30309-3450 (US).

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(54) Title: COMPOSITION AND METHODS FOR CREATING SYNGENEIC RECOMBINANT VIRUS-PRODUCING CELLS

(57) Abstract

Replication-defective viruses and means for intracellular replication thereof are described which are useful for gene therapy. Human cells can be changed into recombinant replication-defective virus particle-producing cells by the simultaneous delivery to those cells of two different nucleic acids: the first being a replication-defective viral genome, the second being a nucleic acid that complements the viral sequences deleted from the first nucleic acid so as to result in the production of new infective virus. The first nucleic acid can be delivered by the replication-defective virus itself or, as a nucleic acid that is not part of the virus. In a preferred embodiment, the replication-defective virus includes elements to maintain the two nucleic acids in combination during transduction. Examples of preferred viral sources are adenoviruses, herpesvirus, retroviruses, and adeno-associated viruses. Nucleic acids useful for gene therapy include those that code for proteins used to identify cells infected with the recombinant virus, those that encode for proteins that function to kill cells containing the viral genome, or that encode for therapeutic proteins that will serve to treat a pathophysiologic condition within the body.

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MG	Madagascar
ML	Mali
MN	Mongolia
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VN	Viet Nam

INTERNATIONAL SEARCH REPORT

Inter. ...nal Application No
PCT/US 95/03729

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/86 C12N5/10 C12N7/04 C07K14/075 C07K14/15
A61K48/00

According to International Patent Classification (IPC) or to both national classification and IPC

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Minimum documentation searched (classification system followed by classification symbols)
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Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X Y	PROC.NATL.ACAD.SCI.USA, vol. 85, 1988, pages 6460-6464, XP002002814 DANOS, O. AND MULLIGAN, R.C.: "Safe and efficient generation of recombinant retroviruses with amphotropic and ecotropic host ranges" see the whole document --- -/--	1,5, 8-12, 16-18,22 2-4,6,7, 13-15, 19-21,23

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[X] Patent family members are listed in annex.

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Date of the actual completion of the international search	Date of mailing of the international search report
10 May 1996	23.05.96
Name and mailing address of the ISA European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl, Fax (+ 31-70) 340-3016	Authorized officer Donath, C

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INTERNATIONAL SEARCH REPORT

International Application No
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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	HUMAN GENE THERAPY, vol. 5, November 1994, pages 1341-1348, XP002002815 GOLDSMITH, K.T. ET AL.: "Trans Complementation of an E1A-Deleted Adenovirus with Codelivered E1A Sequences to Make Recombinant Adenoviral Producer Cells" see the whole document ---	1-23
Y	PROC.NATL.ACAD.SCI.USA, vol. 89, July 1992, pages 6099-6103, XP002002816 WAGNER, E. ET AL.: "Coupling of adenovirus to transferrin-polylysine/DNA complexes greatly enhances receptor-mediated gene delivery and expression of transfected genes" see the whole document ---	1-23
Y	WO,A,93 19092 (CENTRE NATIONAL DE LA RECHERCHE SCIENTIFIQUE) 30 September 1993 see page 4, line 6 - page 8, line 14 see page 9, line 14 - line 26 ---	1-23
Y	WO,A,93 07283 (BOEHRINGER INGELHEIM INTERNATIONAL GMBH) 15 April 1993 see the whole document -----	1-23

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/US 95/03729

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO-A-9319092	30-09-93	AU-B-	1648092	21-10-93
WO-A-9307283	15-04-93	AU-B-	2652692	03-05-93
		BG-A-	98718	28-02-95
		BR-A-	9206559	08-11-94
		CA-A-	2118816	31-03-93
		CZ-A-	9400746	17-05-95
		EP-A-	0545016	09-06-93
		EP-A-	0607206	27-07-94
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		HU-A-	71312	28-11-95
		NO-A-	941154	29-03-94
		NZ-A-	244306	26-07-95
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		CN-A-	1070946	14-04-93